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# Cre-*lox*-Based System for Multiple Gene Deletions and Selectable-Marker Removal in *Lactobacillus plantarum*▽

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**The classic strategy to achieve gene deletion variants is based on double-crossover integration of nonreplicating vectors into the genome. In addition, recombination systems such as Cre-*lox* have been used extensively, mainly for eukaryotic organisms. This study presents the construction of a Cre-*lox*-based system for multiple gene deletions in *Lactobacillus plantarum* that could be adapted for use on gram-positive bacteria. First, an effective mutagenesis vector (pNZ5319) was constructed that allows direct cloning of blunt-end PCR products representing homologous recombination target regions. Using this mutagenesis vector, double-crossover gene replacement mutants could be readily selected based on their antibiotic resistance phenotype. In the resulting mutants, the target gene is replaced by a *lox66*-$P_{32}$-*cat*-*lox71* cassette, where *lox66* and *lox71* are mutant variants of *loxP* and $P_{32}$-*cat* is a chloramphenicol resistance cassette. The *lox* sites serve as recognition sites for the Cre enzyme, a protein that belongs to the integrase family of site-specific recombinases. Thus, transient Cre recombinase expression in double-crossover mutants leads to recombination of the *lox66*-$P_{32}$-*cat*-*lox71* cassette into a double-mutant *loxP* site, called *lox72*, which displays strongly reduced recognition by Cre. The effectiveness of the Cre-*lox*-based strategy for multiple gene deletions was demonstrated by construction of both single and double gene deletions at the *melA* and *bsh1* loci on the chromosome of the gram-positive model organism *Lactobacillus plantarum* WCFS1. Furthermore, the efficiency of the Cre-*lox*-based system in multiple gene replacements was determined by successive mutagenesis of the genetically closely linked loci *melA* and *lacS2* in *L. plantarum* WCFS1. The fact that 99.4% of the clones that were analyzed had undergone correct Cre-*lox* resolution emphasizes the suitability of the system described here for multiple gene replacement and deletion strategies in a single genetic background.**

The development of tools for genetic engineering of gram-positive bacteria is highly valuable for research applications. The classic strategy for obtaining gene deletion variants is based on homologous recombination, using double-crossover integration of heterologous nonreplicating vectors such as pUC (34, 36, 37, 42), pACYC184 (6, 48, 50), or their derivatives in the genome. Several convenient systems that derive from this strategy use conditionally replicating vectors, such as the thermosensitive $pG^+$host system (40) and the RepA-dependent lactococcal pORI system (originating from pWV01) (12, 33, 51) and its broad-host-range derivative (42, 51).

In addition to systems that derive from the classic strategy, various site-specific recombination systems such as Flp-*FRT* (52), Gateway (Invitrogen), ParA-*res* (31), TnpR-*res* (13), and Cre-*lox* are used in mutational strategies. To date, however, these systems have not been available for construction of an unlimited number of mutations in the same genetic background in gram-positive bacteria. For this purpose, the versatile Cre-*lox* system is a promising candidate. The Cre recombinase is a 38-kDa protein that belongs to the integrase family of site-specific recombinases. It catalyzes cofactor-independent recombination between two of its recognition sites, called *loxP*. The 34-bp consensus for *loxP* sites consists of an asymmetrical core spacer of 8 bp, defining the orientation of the *loxP* site, and two 13-bp palindromic flanking sequences (1, 23). A DNA sequence that is flanked by *loxP* sites is excised when the *loxP* sites are convergently oriented, whereas the sequence is inverted when the *loxP* sites are divergently oriented. Cre recombinase is able to act on both inter- and intramolecular *loxP* sites, although recombination of intramolecular *lox* sites is kinetically favorable (32).

The versatile properties of Cre recombinase make it ideal for use in many genetic manipulation strategies. Therefore, the Cre-*lox* system has been used for a wide variety of eukaryotes such as plants (20), *Saccharomyces cerevisiae* (54), mice (45, 55), feline cell lines (30), human cell lines (26, 43), and chicken cell lines (5). For example, recombination of intermolecular *loxP* sites has been used for site-specific integration of transfected DNA into the chromosome (4, 29, 30). Many strategies use recombination of *loxP* sites to excise the intermediate DNA sequence. This includes work on conditional gene deletions (5) and recombinatorial activation of gene expression (55). In particular, an important application of the Cre-*lox* system is selectable-marker excision in gene replacements. Commonly used gene replacement strategies result in the introduction of a selectable marker into the genome, facilitating the selection of gene mutations that might cause growth retardation. However, the expression of the marker may result in polar effects on the expression of genes located upstream and downstream. Selectable-marker removal from the genome by Cre-*lox* recombination is an elegant and efficient way of circumventing this issue and has therefore

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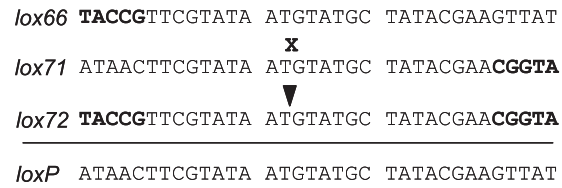


FIG. 1. Schematic representation of mutant *lox66* and *lox71* sites, which after Cre recombination result in a double-mutant *lox72* site. Boldfaced sequences are mutated compared to the native *loxP* site (shown as a reference).

been used frequently, for example, for plants (20), mouse cell lines (2), and yeast (22).

Notably, use of native *loxP* sites for consecutive rounds of gene replacement and subsequent selectable-marker removal would lead to the integration into the genome of multiple *loxP* sites that can still be recognized by Cre. To minimize genetic instability, *lox* sites containing mutations within the inverted repeats (*lox66* and *lox71* [Fig. 1]) have been used for plants (4) and chicken cell lines (5). Recombination of *lox66* and *lox71* results in a *lox72* site that shows strongly reduced binding affinity for Cre, allowing for repeated gene deletion in a single genetic background.

In contrast to the many eukaryotic examples, the Cre-*lox* system has been used much less frequently for prokaryotic organisms. For example, mechanistic studies on Cre recombination have been performed on *Escherichia coli* (3, 44). The Cre-*lox* system was used for conditional gene deletions in *Lactobacillus plantarum* in the murine gastrointestinal tract (10). In addition, Cre-*lox*-mediated selectable-marker removal in gene replacements has been used for the gram-negative bacteria *Methylobacterium extorquens*, *Burkholderia fungorum*, *Escherichia coli*, and *Pseudomonas aeruginosa* (41, 46, 49). However, these experiments used native *loxP* sites for multiple gene replacements. Previously, it was shown for *Lactococcus lactis*, *Corynebacterium glutamicum*, and *Salmonella enterica* serovar Typhimurium that Cre readily excises or inverts large fragments of DNA flanked by *loxP* sites in prokaryotes (14, 15, 57, 62), thereby leading to genomic instability.

Here we describe the construction of a Cre-*lox*-based toolbox for multiple gene deletions in a single genetic background in gram-positive bacteria, a system that combines the advantages of selectable gene replacement and a marker-free, in-frame gene deletion in the final strain. For this purpose, a mutagenesis vector was constructed and used for classic double-crossover replacement of target genes by the selectable-marker cassette *lox66*-$P_{32}$-*cat*-*lox71*, which can be recombined from the chromosome into a double-mutant *lox72* site by transient Cre recombinase expression from a second plasmid.

To validate the effectiveness of the system described here, the mutagenesis targets *melA*, *bsh1*, and *lacS2* were selected in the model organism *L. plantarum* WCFS1, whose genome has been sequenced (28). The *melA* and *bsh1* genes are genetically unlinked loci, whereas the *melA* and *lacS2* loci are closely linked genetically. Although this system has been experimentally tested only for this bacterium, the simple, functional implementation of the same basic characteristics for other bacterial hosts will be discussed.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and primers.** The bacterial strains, plasmids, and primers used in this study and their relevant features are listed in Tables 1 and 2.

As a model strain for gram-positive bacteria, *L. plantarum* WCFS1 (28) was used. *L. plantarum* was grown at 37°C in MRS broth (Difco, West Molesey, United Kingdom) without aeration. *Escherichia coli* strains DH5α (63) and MC1061 (16, 61) were used as intermediate cloning hosts and were grown at 37°C in TY broth (27) with aeration (53). When appropriate, antibiotics were added to the media. For *L. plantarum*, 10 μg/ml chloramphenicol and 10 μg/ml or (for replica plating) 30 μg/ml erythromycin were used. For *E. coli*, 10 μg/ml chloramphenicol and 250 μg/ml erythromycin were used.

**DNA manipulations.** Plasmid DNA was isolated from *E. coli* on a small scale using the alkaline lysis method (7). Large-scale plasmid DNA isolations were performed using Jetstar columns as recommended by the manufacturer (Genomed GmbH, Bad Oberhausen, Germany). For DNA manipulations in *E. coli*, standard procedures were used (53).

*L. plantarum* DNA was isolated and transformed as described previously (25), with slight modifications. For DNA isolation, an overnight culture of *L. plantarum* WCFS1 was diluted 20 times in 50 ml of fresh MRS medium and cells were grown to an optical density at 600 nm ($OD_{600}$) of 1. Cells were pelleted by centrifugation for 10 min at 4,500 rpm (Megafuge 1.0R; Heraeus, Hanau, Germany), resuspended in 2.5 ml of THMS buffer (30 mM Tris-HCl [pH 8], 3 mM $MgCl_2$, 0.73 M sucrose) containing 50 mg/ml lysozyme, and incubated for 2 h at 37°C. Cells were pelleted by centrifugation and resuspended in 2.5 ml Tris-EDTA containing RNase. Subsequently, 125 μl of 10% sodium dodecyl sulfate was added, and cells were incubated for 15 min at 37°C. Then 25 μl of 20-mg/ml proteinase K was added, and the solution was subjected to phenol-chloroform extraction three times. The total DNA was precipitated with isopropanol, washed with 70% ethanol, dried, and taken up in water. For transformation of *L. plantarum* WCFS1, a preculture in MRS broth was diluted in MRS broth containing 1% glycine and cells were grown to an $OD_{600}$ of 1. Cells were kept on ice for 10 min and pelleted by centrifugation for 10 min at 4,000 rpm (Megafuge 1.0R; Heraeus, Hanau, Germany). Cells were then resuspended in ice-cold 30% polyethylene glycol 1450 and kept on ice for 10 min. Finally, cells were pelleted by centrifugation for 10 min at 4,000 rpm and concentrated 100-fold into ice-cold 30% polyethylene glycol 1450. Subsequently, 40 μl of the cell suspension and a maximum of 5 μl of the plasmid DNA solution were electroporated using a GenePulser Xcell electroporator (Bio-Rad, Veenendaal, The Netherlands) in cuvettes with a 2-mm electroporation gap at 1.5 kV, 25 μF capacitance, and 400 Ω parallel resistance.

Restriction endonucleases, *Taq*, *Pfx*, and *Pwo* DNA polymerases, T4 DNA ligase, and Klenow enzyme were used as specified by the manufacturers (Promega, Leiden, The Netherlands; Boehringer, Mannheim, Germany). Primers were obtained from Genset Oligos (Paris, France).

**Mutagenesis vector construction.** To facilitate construction of chromosomal gene replacements, the universal mutagenesis vector pNZ5319 was constructed (Fig. 2A). For this purpose, the pACYC184-derived origin of replication was amplified by PCR (using *Pfx* polymerase, primers pNZ84F and pNZ84R, and pNZ84 [59] as template DNA) and cloned into the NaeI restriction site of pGIZ850 (21), resulting in pNZ7101 (8). To introduce *lox66* and blunt-end restriction sites SwaI and PmeI, 80 and 81 linkers (Table 2) were annealed and cloned into the Bsp1286I and *Tth*111I restriction sites upstream of the $P_{32}$-*cat* cassette of pNZ7101 (8), yielding pNZ5315 (Table 1). Subsequently, 82 and 83 linkers (Table 2), which contained *lox71* and blunt-end restriction sites Ecl136II and SrfI, were annealed and cloned into the PpuMI and PvuI restriction sites downstream of the $P_{32}$-*cat* cassette of pNZ5315, yielding pNZ5317 (Table 1). Furthermore, both the *las* operon and *pepN* terminator regions (38, 39) were amplified by PCR from *L. lactis* MG1363 (19) using primers LasTermi_F and LasTermi_R and primers 111 and 113, respectively (Table 2). The PCR fragment containing the *las* terminator and the BglII restriction site was digested with Ecl136II (to avoid introduction of an extra Ecl136II restriction site into the mutagenesis vector) and cloned into pNZ5317, which had been digested with AflIII and treated with Klenow enzyme to generate blunt ends. Subsequently, the PCR fragment containing the *pepN* terminator and the XhoI restriction site was cloned into the PvuII restriction site of the targeting vector, yielding pNZ5318 (Table 1). Residual and nonfunctional DNA sequences were removed from the pNZ5318 mutagenesis vector by BbsI and SalI digestion, treatment with Klenow enzyme to generate blunt ends, and self-ligation, yielding pNZ5319 (Table 1).

**Construction of gene-specific mutagenesis vectors.** For construction of gene-specific mutagenesis vectors, a standard cloning procedure was used (Fig. 2B). Typically, a 1-kb fragment of the upstream sequence and a 1-kb fragment of the downstream sequence of the target locus were amplified by PCR using a proof-

TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Relevant feature(s)[a] | Reference(s) |
|---|---|---|
| Strains | | |
| *L. plantarum* | | |
| WCFS1 | Single-colony isolate of *L. plantarum* NCIMB8826 | 28 |
| NZ5304 | Derivative of WCFS1 containing a *lox66*-$P_{32}$-*cat*-*lox71* replacement of *bsh1* (*bsh1*:: *lox66*-$P_{32}$-*cat*-*lox71*) | This work |
| NZ5305 | Derivative of NZ5304 containing a *lox72* replacement of *bsh1* (Δ*bsh1*) | This work |
| NZ5334 | Derivative of WCFS1 containing a *lox66*-$P_{32}$-*cat*-*lox71* replacement of *melA* (*melA*:: *lox66*-$P_{32}$-*cat*-*lox71*) | This work |
| NZ5335 | Derivative of NZ5334 containing a *lox72* replacement of *melA* (Δ*melA*) | This work |
| NZ5337 | Derivative of NZ5335 containing a *lox72* replacement of *melA* and *bsh1* (Δ*melA* Δ*bsh1*) | This work |
| NZ5338 | Derivative of NZ5335 containing a *lox66*-$P_{32}$-*cat*-*lox71* replacement of part of *lacS2* (Δ*melA* *lacS2*::*lox66*-$P_{32}$-*cat*-*lox71*) | This work |
| NZ5339 | Derivative of NZ5338 containing a *lox72* replacement of *melA* and part of *lacS2* (Δ*melA* Δ*lacS2*) | This work |
| *E. coli* | | |
| DH5α | Cloning host; F′ ϕ80d*lacZ*Δ*M15 endA1 recA1 hsdR17* ($r_K^-$ $m_K^+$) *supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169 deoR* $\lambda^-$ | 63 |
| MC1061 | Cloning host; $F^-$ *araD139* Δ(*ara-leu*)*7696 galE15 galK16* Δ(*lac*)*X74 rpsL* ($Str^r$) *hsdR2* ($r_K^-$ $m_K^+$) *mcrA* *mcrB1* | 16, 61 |
| *L. lactis* MG1363 | Plasmid-free derivative of NCDO 712 | 19 |
| Plasmids | | |
| pCR-Blunt | $Kan^r$; cloning vector for blunt-end PCR products | Invitrogen |
| pGID023 | $Em^r$; pJDC9 derivative containing the pE194 replication functions; unstable in lactobacilli | 24 |
| pGIZ850 | $Cm^r$ $Em^r$ $Ap^r$; pUC18 derivative containing a $P_{32}$-*cat* cassette that is selectable at the single-copy level | 21 |
| pNZ273 | $Cm^r$; pNZ124 carrying the promoterless *gusA* gene from *E. coli* | 47 |
| pNZ5315 | $Cm^r$ $Em^r$; pNZ7101 derivative containing a *lox66* site | This work |
| pNZ5317 | $Cm^r$ $Em^r$; pNZ5315 derivative containing a *lox71* site | This work |
| pNZ5318 | $Cm^r$ $Em^r$; pNZ5317 derivative for multiple gene replacements containing *las* and *pepN* terminators | This work |
| pNZ5319 | $Cm^r$ $Em^r$; pNZ5318 derivative for multiple gene replacements in gram-positive bacteria | This work |
| pNZ5340 | $Cm^r$ $Em^r$; pNZ5319 derivative containing homologous regions up- and downstream of WCFS1 *melA* | This work |
| pNZ5325 | $Cm^r$ $Em^r$; pNZ5319 derivative containing homologous regions up- and downstream of WCFS1 *bsh1* | This work |
| pNZ5344 | $Cm^r$ $Em^r$; pNZ5319 derivative containing homologous regions up- and downstream of *lacS2* in the *melA*::*lox72* strain NZ5335 | This work |
| pNZ5346 | $Cm^r$; pNZ273 derivative containing the promoter region and ribosome binding site of lp_1144 of WCFS1 | This work |
| pNZ5347 | $Cm^r$; pNZ7110 derivative containing the promoter region and ribosome binding site of lp_1144, driving expression of *cre* | This work |
| pNZ5348 | $Em^r$; pGID023 derivative containing *cre* under the control of the lp_1144 promoter | This work |
| pNZ7101 | $Cm^r$ $Em^r$; pACYC184 derivative for gene replacements | 8 |
| pNZ7110 | $Ap^r$; pUC18 derivative containing the *cre* gene | 9 |
| pNZ84 | $Cm^r$; pACYC184 derivative | 59 |

[a] $Kan^r$, kanamycin resistant; $Cm^r$, chloramphenicol resistant; $Em^r$, erythromycin resistant; $Ap^r$, ampicillin resistant.

reading polymerase and cloned into the SwaI or PmeI and Ecl136II or SrfI blunt-end restriction sites of pNZ5319, respectively. When desired, XhoI or BglII sticky-end restriction sites can be used in combination with the blunt-end restriction sites. To ultimately obtain in-frame gene deletions (following Cre recombination [see below and Fig. 2C]), primers were designed in such a way that the 5′- and 3′-flanking regions of the target gene encompassed the first and last five codons of that gene, respectively. The efficiency of cloning of the PCR amplification products of the 5′- and 3′-flanking regions of the target gene into the mutagenesis vector was enhanced by removal of the self-ligation vector in the ligation mixture by digestion with SwaI, PmeI, Ecl136II, or SrfI, depending on the restriction site used for cloning. Colonies harboring the anticipated insert in the desired orientation could be identified effectively by colony PCR using a vector-specific primer (annealing to the $P_{32}$-*cat* region; reverse primer 85 for cloning of 5′ sequences into the SwaI or PmeI restriction site and forward primer 87 for cloning of 3′ sequences into the Ecl136II or SrfI restriction site), combined with an insert-specific primer (see also below) (Table 2).

By following the strategy described above, the *melA* mutagenesis vector pNZ5340 was constructed by successive cloning of the 5′- and 3′-flanking regions of *melA* (lp_3485) (28) (Fig. 2B) into the SwaI and Ecl136II restriction sites of pNZ5319 (amplified by PCR using *Pfx*, *L. plantarum* WCFS1 genomic DNA as a template, and primer sets 91-90 and 92-93, respectively [Table 2]). Clones that harbored the anticipated inserts were identified by PCR using primer sets 91-85 and 87-93, respectively (Table 2; Fig. 2B).

Likewise, the *bsh1* replacement vector pNZ5325 was constructed by the successive cloning of PCR products of the 5′- and 3′-flanking regions of WCFS1 *bsh1* (lp_3536) (28) (amplified using *Pfx* polymerase, WCFS1 genomic DNA as a template, and primer sets 101-102 and 103-104, respectively [Table 2]) into pNZ5319 digested with SwaI and Ecl136II, respectively. Clones that harbored the correct inserts were identified by PCR using primer sets 101-85 and 87-104, respectively (Table 2).

The third locus, *lacS2* (lp_3486) (28), was targeted for mutagenesis in a *L. plantarum* WCFS1 Δ*melA* background (NZ5335 [Table 1]). For construction of the corresponding mutagenesis vector pNZ5344, PCR products of 5′ and 3′ regions of *lacS2* (amplified using *Pfx* polymerase, Δ*melA* [NZ5335] template DNA, and primer sets 124-125 and 126-127, respectively [Table 2]) were digested with XhoI and BamHI, respectively, and sequentially cloned into XhoI- and SwaI-digested and BglII- and Ecl136II-digested pNZ5319. Clones harboring the correct insert were identified using primer sets 124-85 and 87-127, respectively (Table 2).

**Mutant construction.** In order to engineer *lox66*-$P_{32}$-*cat*-*lox71* gene replacements, 4 μg of the appropriate mutagenesis vector was transformed into *L. plantarum* WCFS1 by electroporation as described previously (25). Chloramphenicol-resistant ($Cm^r$) transformants were selected and replica plated to check for an erythromycin-sensitive ($Em^s$) phenotype. Candidate double-crossover clones ($Cm^r$ $Em^s$) were analyzed by PCR amplification of the *cat* and *ery* genes using primers cat96F-cat97R and eryintF-eryintR, respectively (Table 2). Correct integration of the *lox66*-$P_{32}$-*cat*-*lox71* cassette into the genome (for *melA* replacement, see Fig. 3A) was confirmed by PCR amplification of the flanking regions

TABLE 2. Primers used in this study

| Primer | Sequence (5′ to 3′) |
|---|---|
| Bsh1fr1F | GATTAAGTTTGCAGGACATGGAG |
| Bsh1R | GCCAGCCATTGGAACTTACTCTG |
| Cat96F | TCAAATACAGCTTTTAGAACTGG |
| Cat97R | ACCATCAAAAATTGTATAAAGTGGC |
| CreF | CTAACTCGAGTGATCACCAATTC |
| CreR | GGCTATCAATCAAAGCAACACG |
| EryintF | CGATACCGTTTACGAAATTGG |
| EryintR | CTTGCTCATAAGTAACGGTAC |
| Lastermi_F | ACGTCCGCGGGGACAATATGGGGTAAGCG |
| Lastermi_R | AAGAAGATCTCTAAAGCTGACGGGGTAAAC |
| MellacF | ACCGTTAAGATGCGTGGGACTGG |
| MellacR | CATAGTAAAATCTTCCCTTCGCTA |
| pNZ84F | CGGGATCCCAACAGTACTGCGATGAG |
| pNZ84R | GGGGTACCATCCAGTGATTTTTTTCTCC |
| 80 | CGTTTAAACAATTTAAATCTACCGTTCGTATA ATGTATGCTATACGAAGTTATGACA |
| 81 | TTGTCATAACTTCGTATAGCATACATTATACG AACGGTAGATTTAAATTGTTTAAACGTGCT |
| 82 | GACCCATAACTTCGTATAATGTATGCTATACG AACGGTACAGCCCGGGCATGAGCTCCGAT |
| 83 | CGGAGCTCATGCCCGGGCTGTACCGTTCGTAT AGCATACATTATACGAAGTTATGG |
| 85 | GTTTTTTTCTAGTCCAAGCTCACA |
| 87 | GCCGACTGTACTTTCGGATCCT |
| 90 | CATAGTAAAATCTTCCCTTCGC |
| 91 | GTCGTAAAGTGTTCTTCTTAGC |
| 92 | GCTAAGGACTAAGCTCAGCC |
| 93 | GAGTTTAGGACTACAGGGGGC |
| 95 | AATATGTGTACAGGCTGAGCTTAGTCCTT AGCC |
| 101 | GATTGCGATTGATATCGATGGC |
| 102 | TATGGCAGTACACATAACTAGTAATCCTCC |
| 103 | TACTATGCAGTTAACTAAAAGCC |
| 104 | CTTACCAATCATGCGTCCCG |
| 106a | GTTCGATAAAGAATGAGGATGGC |
| 107a | TTATCGCAAGTATCTCAAATTGCG |
| 108 | TATTGGCCTTCCCACCATTAGC |
| 109 | CACGTTATTTACGGCGACGGG |
| 111 | CGTGTTGCTTTGATTGATAGCC |
| 113 | GCTCGAGCGCGTTATCGGTCCTTTAATTGG |
| 124 | GCGCCTCGAGCCGCAATCGCTTTGATTTC TGCC |
| 125 | TCCGATTGCATGGCGAGTCGG |
| 126 | TACCGTTAAGATGCGTGGG |
| 127 | GGCGGGATCCTTATACGGTGACAGCAGACGG |
| 128 | CATTAAACCAACGGATGGTCGCG |
| 130 | CGTGGTTGGATGGCATTTGGG |
| 137 | CTTCTACCCATTATTACAGCA |
| 1144F | GGATCCGTCGACCGCGATTTTTGTATGAGATG |
| 1144R | GGATCCGCTGTTCGCCACCCTTTCTA |

of the integrated *lox66*-$P_{32}$-*cat-lox71* cassette using primers annealing uniquely to genomic sequences (for *melA*, primer 108 for amplification of the 5′ region and primer 109 for amplification of the 3′ region) combined with the mutagenesis vector-specific primers 85 and 87, respectively, which annealed to the $P_{32}$-*cat* region (Table 2; Fig. 3A). Likewise, for analysis of the flanking regions of the *lox66*-$P_{32}$-*cat-lox71* replacement of *bsh1*, primers 106a-85 and 87-107a were used; for *lacS2* replacement, primers 130-85 and 87-109 were used (Table 2; Fig. 3A).

**Transient Cre expression vector.** For expression of Cre, a 100-bp region upstream of the *L. plantarum* WCFS1 gene lp_1144 (28), containing the functional promoter $P_{1144}$ (11), was amplified by proofreading PCR using primers 1144F and 1144R and then cloned into pCR-Blunt (Invitrogen, Breda, The Netherlands) (Tables 1 and 2). To assess the promoter activity of the $P_{1144}$ fragment, it was digested from the pCR-Blunt vector with BamHI and cloned upstream of the *gusA* gene into BglII-digested pNZ273 (47), resulting in pNZ5346. Quantitative β-glucuronidase activity measurements were performed as described previously (18). To determine *cre* expression, the promoter fragment was digested from pNZ5346 with SalI and BamHI and then cloned upstream of *cre* into SalI- and BamHI-digested pNZ7110 (9), yielding pNZ5347. Finally, the $P_{1144}$ promoter-*cre* cassette was digested from pNZ5347 using KpnI and HindIII and then cloned into correspondingly digested pGID023, yielding pNZ5348, which is unstable in lactobacilli (24).

The stability of the pGID023 replicon in *L. plantarum* was determined in duplicate by culturing for 10 generations without selection pressure. Subsequently, cells were plated with and without selection pressure, and the CFU count per milliliter was determined. The presence of pGID023 in *L. plantarum* was verified by colony PCR using primers eryintF and eryintR (Table 2).

**Cre-mediated mutant locus resolution.** To excise the $P_{32}$-*cat* selectable-marker cassette from the chromosome, 4 μg of the transient erythromycin-selectable *cre* expression plasmid pNZ5348 was transformed into *lox66*-$P_{32}$-*cat-lox71* gene replacement mutants. After 48 to 72 h of growth, $Em^r$ colonies were checked by PCR for the presence of cells that had undergone Cre-mediated recombination, using primers spanning the recombination locus (specifically, primers 108 and 109 for *melA* [Fig. 3A], bsh1fr1F and bsh1R for *bsh1*, and 128, 137, and 95 in one PCR for *lacS2* [Fig. 3B]) (Table 2). The pNZ5348 vector was cured from appropriate colonies of *L. plantarum* mutants by growth without erythromycin selection pressure for 10 generations. To obtain clonal strains, single-colony isolates were selected for which curing of the Cre expression vector was confirmed by the absence of PCR amplification of *ery* (using primers eryintF and eryintR) and *cre* (using primers creF and creR) (Table 2), and Cre-mediated recombination was confirmed by PCR amplification as described above. Additionally, the presence of a correctly resolved *lox72* site (Fig. 2C) was confirmed by sequencing (Baseclear, Leiden, The Netherlands) using primers 95, bsh1fr1F, and 95 for *melA*, *bsh1*, and *lacS2* replacement, respectively.

**Southern blot analysis.** To confirm the genotype of WCFS1 *melA*::*lox66*-P32-*cat-lox71* (NZ5334), Δ*melA* (NZ5335), Δ*melA lacS2*::*lox66*-P32-*cat-lox71* (NZ5338), and Δ*melA* Δ*lacS2* (NZ5339) mutant derivatives (Table 1), Southern blot analysis was performed as described previously (53) using AvaI and DraI digests of total DNA. As a probe, a PCR amplification product of the intergenic region of *melA* and *lacS2* (amplified with *Taq* polymerase, WCFS1 total DNA, and primers mellacF and mellacR) was used.

**HPLC assay of bile salt hydrolase activity.** To determine the bile salt hydrolase activity of *L. plantarum*, an overnight culture was inoculated 1:10 into fresh MRS medium and cells were grown to an $OD_{600}$ of 5. Cells were pelleted by centrifugation for 10 min at 4,500 rpm (Megafuge 1.0R; Heraeus, Hanau, Germany) and resuspended in MRS medium to an $OD_{600}$ of 100. For determination of bile salt hydrolase activity, wild-type *L. plantarum* cells were diluted in MRS to an $OD_{600}$ of 10, whereas cells of *bsh1* deletion strains were used undiluted. Conversion of the bile salt glycocholic acid (Sigma, Zwijndrecht, The Netherlands) was determined by high-performance liquid chromatography (HPLC) as described previously (17). Separations were carried out with a reversed-phase resin-based column (PLRP-S; 5-μm particles; 300-Å pore size; 250-mm column length; 4.6-mm inner diameter; Polymer Laboratories, Shropshire, United Kingdom) and a matching precolumn. Bile salts were detected using a pulsed amperometric detector (EG&G Princeton Applied Research, Princeton, NJ) equipped with a gold working electrode and a reference electrode (Ag/AgCl).

**Nucleotide sequence accession number.** The sequence of the pNZ5319 mutagenesis vector is available in the GenBank database under accession number DQ104847. The sequence of the $P_{1144}$-*cre* cassette of the *cre* expression plasmid pNZ5348 is available in the GenBank database under accession number DQ340306.

## RESULTS

**Strategy of gene replacement and selectable-marker removal.** For generation of gene deletions, the mutagenesis vector pNZ5319 (Fig. 2B) was constructed and implemented in the gram-positive model organism *Lactobacillus plantarum* WCFS1 (28). This medium-copy-number *E. coli* cloning vector contains a PACYC184 origin of replication, which is suitable for "suicide" mutagenesis in lactic acid bacteria, as described previously (18, 59).

Cloning of PCR-amplified homologous DNA fragments upstream and downstream of the mutagenesis locus (necessary for targeting of the mutagenesis vector to the genomic locus) in pNZ5319 is facilitated by the presence of PmeI, SwaI, SrfI, and Ecl136II rare-cutting blunt-end restriction sites in the vector, flanked by the lactococcal *las* (38, 39) and *pepN* (58) terminators (Fig. 2B).

Furthermore, the mutagenesis vector pNZ5319 contains both a chloramphenicol (*lox66*-$P_{32}$-*cat-lox71*) and an erythro-

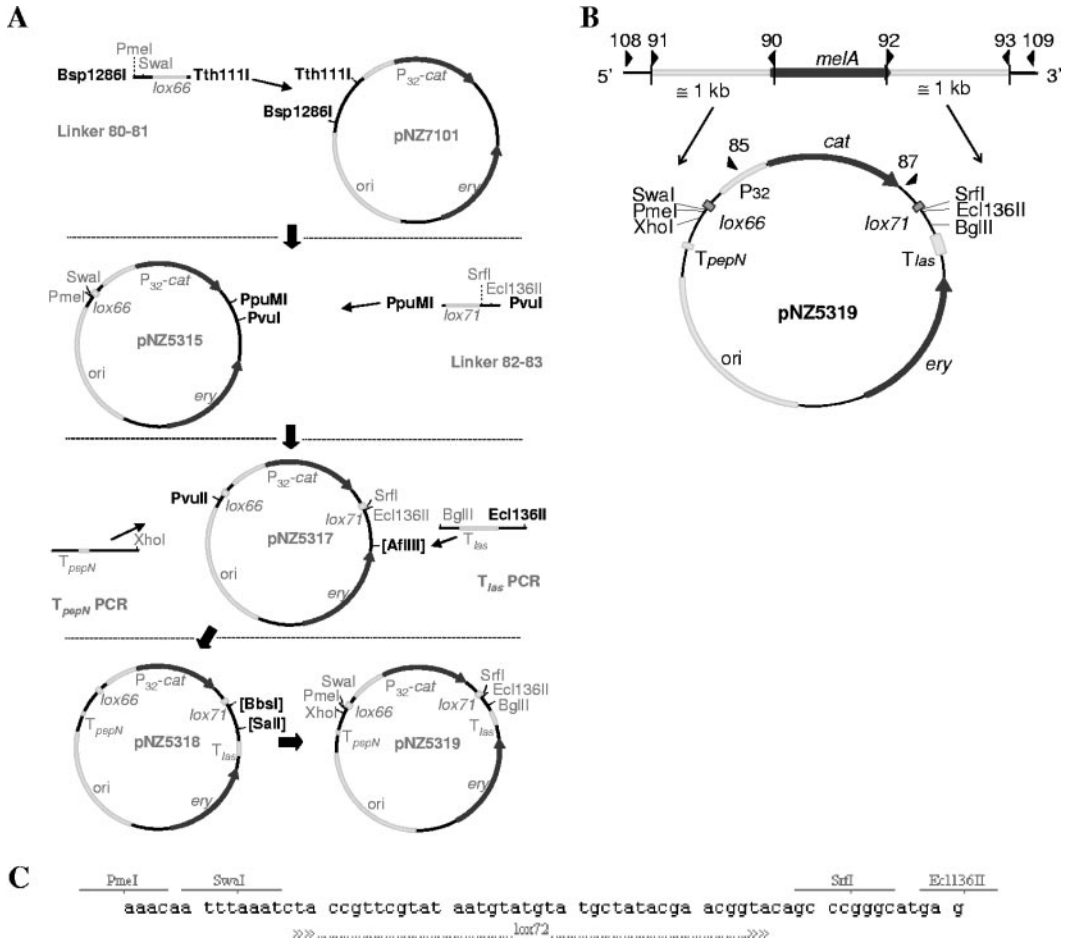


FIG. 2. (A) Schematic representation of the construction of mutagenesis vector pNZ5319. The 80 and 81 linkers were annealed (Table 2) and cloned into pNZ7101 digested with Bsp1286I and *Tth*111I, yielding pNZ5315 (Table 1). Subsequently, the 82 and 83 linkers (Table 2) were annealed and cloned into pNZ5315 digested with PpuMI and PvuI, yielding pNZ5317 (Table 1). Then the *las* terminator (38, 39) and *pepN* terminator (58) were amplified by PCR from *L. lactis* MG1363 (19) using primers LasTermi_F and LasTermi_R and primers 111 and 113, respectively (Table 2). The PCR fragment containing the *las* terminator and the BglII restriction site was digested with Ecl136II, cloned into pNZ5317 digested with AflIII, and treated with Klenow enzyme. Subsequently, the PCR fragment containing the *pepN* terminator and the XhoI restriction site was cloned into the PvuII site of the targeting vector, yielding pNZ5318 (Table 1). Finally, nonfunctional DNA sequences were removed by BbsI and SalI digestion, treatment with Klenow enzyme, and self-ligation, resulting in pNZ5319 (Table 1). (B) Schematic representation of mutagenesis vector pNZ5319. Indicated are the pACYC184-derived origin of replication (ori), the erythromycin resistance gene (*ery*), the chloramphenicol resistance gene under the control of the $P_{32}$ promoter ($P_{32}$-*cat*), flanked by *lox66* and *lox71* sites, and the lactococcal $T_{las}$ and $T_{pepN}$ terminators. The presence of rare-cutting blunt-end restriction sites SwaI, PmeI, SrfI, and Ecl136II allows direct cloning of blunt-end PCR products of the flanking regions of the target locus. As an example, the regions used for PCR amplification and cloning into pNZ5319 for construction of a *L. plantarum melA* mutant are indicated. In addition, the sticky-end restriction sites XhoI and BglII can be used in combination with the blunt-end restriction sites. The presence of two selectable-marker gene cassettes ($P_{32}$-*cat* and *ery*) on the mutagenesis vector allows direct selection of double-crossover integrants based on their antibiotic resistance ($Cm^r$) and sensitivity ($Em^s$) phenotype. Black arrowheads indicate primers used in this study. (C) Schematic representation of the in-frame insertion that is left in the genome after *lox72* replacement of the target gene. Depending on the restriction sites used for cloning of the homologous DNA fragments (as indicated in the figure) that encompass a whole number of codons of the target gene, the number of foreign nucleotides left in the genome is 45 (cloning using SwaI and SrfI restriction sites), 54 (PmeI/SrfI or SwaI/Ecl136II), or 63 (PmeI/Ecl136II), thereby creating an in-frame deletion of the target gene.

mycin (*ery*) resistance cassette that can be selected at the single-copy chromosomal level. Following transformation of the mutagenesis vector to the target organism, the antibiotic resistance cassettes allow for direct selection of double-crossover mutants on the basis of their antibiotic resistance and sensitivity phenotype. In the resulting double-crossover mutant strains, the target gene is replaced by a *lox66*-$P_{32}$-*cat*-*lox71* cassette. The presence of the *lox* sites renders the $P_{32}$-*cat* cassette excisable from the genome of the double-crossover mutant strain by Cre recombinase. Using native *loxP* sites, multiple gene deletions would lead to the integration into the genome of multiple *loxP* sites that can cause genomic instability in the presence of Cre (14, 15, 57). Therefore, *loxP* sites containing mutations within the inverted repeats (*lox66* and *lox71*) (4) were used (Fig. 1); after Cre recombination, this strategy results in a double-mutant *loxP* site (*lox72*), which shows strongly lowered affinity for Cre.

Transient Cre expression was driven by $P_{1144}$ (upstream of the lp_1144 gene) from the pNZ5348 vector, which contains a replicon that is unstable in lactobacilli (24). Although this

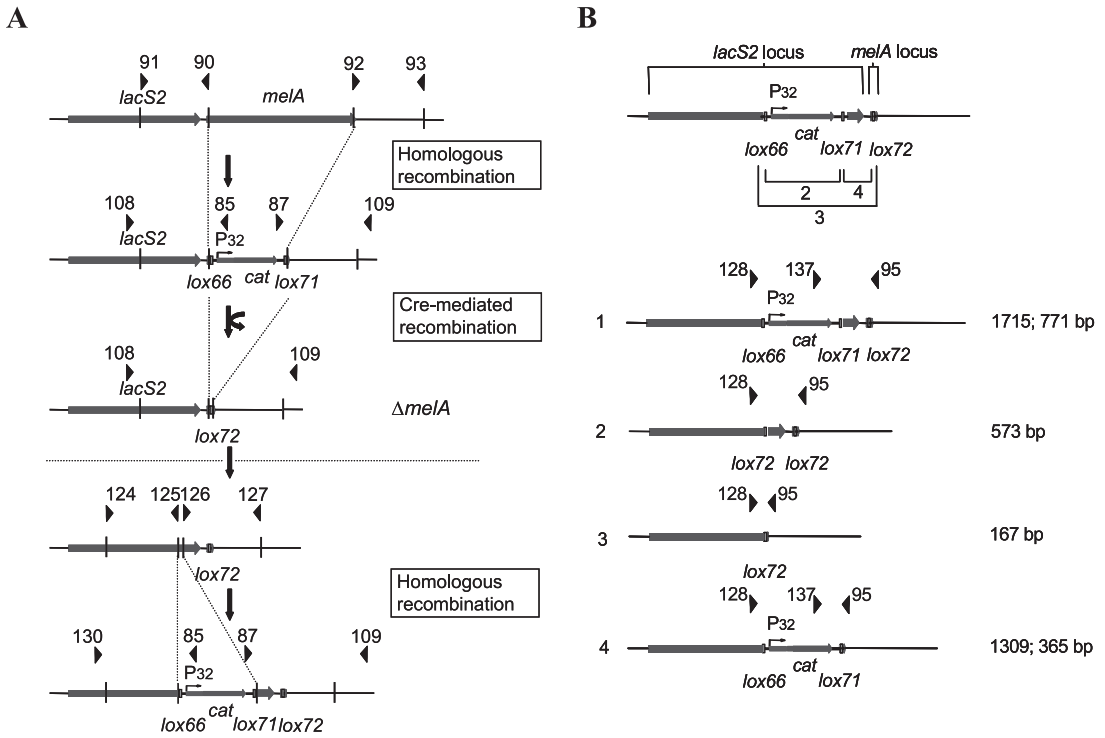


FIG. 3. (A) Strategy for construction of double-crossover mutants and subsequent Cre-*lox*-mediated selectable-marker removal (*melA* and subsequent *lacS2* mutagenesis are shown as an example). After transformation of the gene-specific mutagenesis vector, the target gene (*melA*) is replaced by a *lox66*-$P_{32}$-*cat*-*lox71* cassette by homologous recombination. After selection of the double-crossover mutant, the *lox66*-$P_{32}$-*cat*-*lox71* cassette is resolved to a single double-mutant *lox72* site by transient Cre expression from a curable plasmid. Subsequently, the next round of gene replacement (*lacS2*) can be performed. Black arrowheads indicate primers that were used for amplification of the homologous DNA fragments used for targeting cloning in the pNZ5319 mutagenesis vector (primers 91 and 90 and primers 92 and 93 for *melA* targeting; primers 124 and 125 and primers 126 and 127 for *lacS2* targeting), confirmation of correct integration of the mutagenesis vector into the genome (primers 108 and 85 and primers 87 and 109 for *melA* targeting; primers 130 and 85 and primers 87 and 109 for *lacS2* targeting), and confirmation of Cre resolution of the *lox66*-$P_{32}$-*cat*-*lox71* cassette (primers 108 and 109 for *melA* targeting). (B) Possible products of Cre recombination during multiple gene replacement in a single genetic background, as exemplified by recombination of *lox66*-$P_{32}$-*cat*-*lox72* at the *lacS2* locus in a Δ*melA* background. Black arrowheads indicate primers that were used to distinguish the four possible products of *lacS2*::*lox66*-$P_{32}$-*cat*-*lox72* recombination in a Δ*melA* background by PCR (primers 128, 137, and 95 in one reaction mixture). The corresponding PCR product sizes are given on the right. (Diagram 1) No recombination occurred. (Diagram 2) Correct recombination occurred, removing the $P_{32}$-*cat* selectable marker cassette. (Diagram 3) Incorrect recombination between *lox66* and *lox72* occurred, resulting in deletion of the intermediate region. (Diagram 4) Incorrect recombination between *lox71* and *lox72* occurred, resulting in deletion of the intermediate region.

plasmid can be introduced into *L. plantarum* when selective ($Em^r$) conditions are maintained, its intrinsic instability in this host resulted in rapid curing of the plasmid when selective pressure was relieved. In our experiments, a ca. 1,000-fold reduction in plasmid retention was obtained by culturing for 10 generations in the absence of selection pressure, as determined by antibiotic resistance profiling and PCR (data not shown). Finally, the $P_{1144}$ promoter is predicted to drive constitutive, moderate levels of transcription of the downstream gene (*pcrA*), encoding a DNA helicase. Previous experiments in our laboratory using *gusA* (which encodes β-glucuronidase) as a promoter probe confirmed the prediction of the characteristics of the $P_{1144}$ promoter (data not shown).

Notably, in our design, the PCR-amplified homologous DNA fragments used for locus targeting include a number of complete codons of the 5′ and 3′ ends of the gene targeted for mutagenesis. Thus, depending on the restriction sites in pNZ5319 that were used for cloning of the 5′ and 3′ homologous regions, the total number of foreign nucleotides left in the genome after Cre-mediated excision of the selectable-marker cassette is 45 (using SrfI and SwaI), 54 (using SrfI and PmeI or Ecl136II and SwaI), or 63 (using Ecl136II and PmeI), thereby generating an in-frame deletion (Fig. 2C).

**Single-locus mutagenesis.** To validate the gene deletion system, the *melA* gene (lp_3485) and the *bsh1* gene (lp_3536) of *L. plantarum* WCFS1 (28) were chosen as target genes for single-locus mutagenesis. The *melA* gene encodes an α-galactosidase (EC 3.2.1.22), which is predicted to be involved in hydrolysis of the sugar melibiose into galactose and glucose. In *L. plantarum*, the *melA* gene is induced by melibiose and repressed by glucose (56). The *melA* gene is a convenient target for mutagenesis, since it is predicted to encode a nonredundant function in *L. plantarum* WCFS1 and its phenotype is likely to be measurable both quantitatively (by hydrolysis of a chromogenic substrate) and qualitatively (by the absence of growth on melibiose as a sole carbon source). The *bsh1* gene of *L. plantarum* WCFS1 is predicted to encode a bile salt hydrolase (Bsh; EC 3.5.1.24). Bile salt hydrolases catalyze cleavage of the amino acid moiety from the steroid nucleus of conjugated bile salts. The DNA sequence of *L. plantarum* WCFS1

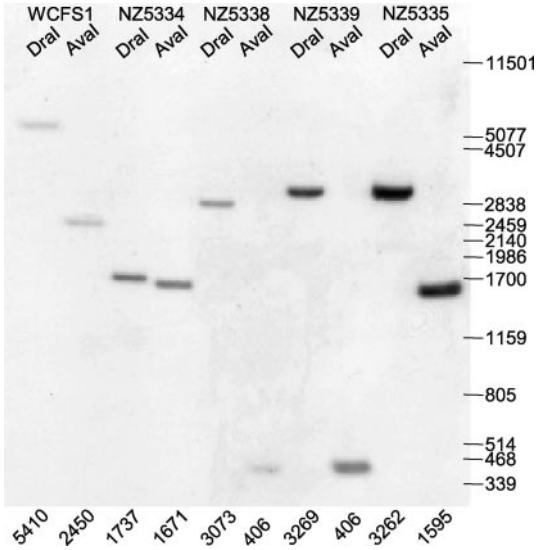


FIG. 4. Southern blot analysis of WCFS1 and its *melA*::*lox66*-$P_{32}$-*cat-lox71* (NZ5334), Δ*melA lacS2*::*lox66*-$P_{32}$-*cat-lox71* (NZ5338), Δ*melA* Δ*lacS2* (NZ5339), and Δ*melA* (NZ5335) derivatives. The restriction enzymes (DraI and AvaI, respectively) used for restriction of genomic DNA of the strains were chosen in such a way that all strains could be distinguished based on the predicted sizes of the hybridizing bands (given below the blot in base pairs). The sizes of the hybridizing bands of the various strains were as expected (compare the predicted sizes with the positions of the bands relative to the DNA ladder on the right). DraI, DraI digestion of total DNA; AvaI, AvaI digestion of total DNA.

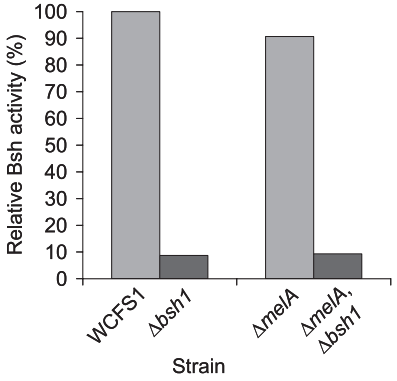


FIG. 5. Bsh activities of Δ*bsh1* (NZ5303), Δ*melA* (NZ5335), and Δ*melA* Δ*bsh1* (NZ5337) strains relative to that of WCFS1 (taken as 100%). Deletion of *bsh1* in WCFS1 or NZ5335 results in a 90% reduction in Bsh activity.

*bsh1* is 99% identical to the sequence of the *bsh* gene of *L. plantarum* LP80, for which a previously constructed *bsh* mutant derivative was shown to be deficient in bile salt hydrolase activity (35). However, the *L. plantarum* WCFS1 genome appears to be fourfold redundant for this function, containing genes annotated as *bsh1* to *bsh4* (28). The *L. plantarum bsh1* gene was selected as a target for mutagenesis in order to investigate whether it is the sole bile salt hydrolase-encoding gene in this strain.

For construction of *melA* and *bsh1* mutant strains, gene-specific mutagenesis vectors pNZ5340 and pNZ5325 were constructed by direct cloning of PCR-amplified homologous DNA fragments upstream and downstream of *melA* or *bsh1*, respectively, and transformed to *L. plantarum* WCFS1. The genetic events involved are schematically illustrated for the *melA* locus in Fig. 3A. During primary selection of *melA* double-crossover mutants, 34 $Cm^r$ colonies were found, 9 of which appeared to display the $Em^s$ phenotype correlating to the *melA*::*lox66*-$P_{32}$-*cat-lox71* genotype. A single-colony isolate was selected and designated NZ5334 (Table 1). For *bsh1* mutagenesis, a slightly lower number of $Cm^r$ colonies, 12, was found during primary selection of double-crossover mutants; the proportion of colonies displaying an $Em^s$ phenotype (correlating to a *bsh1*::*lox66*-$P_{32}$-*cat-lox71* genotype), 3 of 12, appeared to be similar to that observed for the *melA* locus. A single-colony isolate was selected and designated NZ5304 (Table 1). Correct integration of the *lox66*-$P_{32}$-*cat-lox71* cassette into the genome was confirmed by PCR and by Southern blot analysis (Fig. 4).

To resolve the *lox66*-$P_{32}$-*cat-lox71* cassette at the mutation locus to a single in-frame *lox72* site, the erythromycin-selectable Cre expression plasmid pNZ5348 was transformed to the NZ5334 and NZ5304 double-crossover mutant strains. After 48 h of incubation, 22 $Em^s$ colonies of each strain were analyzed, of which 9 (for the *melA*-mutagenized strain) and 15 (for the *bsh1*-mutagenized strain) colonies were found to contain cells that had undergone Cre recombination, as determined by PCR. Subsequently, the Cre expression vector was allowed to be lost from the cells by culturing without selective pressure. Single-colony isolates (designated NZ5335 [Δ*melA*] and NZ5305 [Δ*bsh1*] [Table 1]) were selected and analyzed for loss of the Cre expression vector and correct Cre-mediated recombination of the target loci, as determined by an $Em^s$ phenotype, PCR (Fig. 3A), DNA sequencing of the mutated locus, and Southern blot analysis (Fig. 4).

To further confirm the genotypes of these mutants, the anticipated physiological consequences were analyzed. As expected, growth of the *L. plantarum* WCFS1 *melA* deletion mutant NZ5335 on MRS plates in the presence of glucose as a carbon and energy source was unaffected compared to growth of the wild type. However, in the presence of melibiose as the sole carbon and energy source, growth of the *L. plantarum* WCFS1 *melA* deletion mutant NZ5335 on MRS plates was completely abolished, whereas growth of the wild-type strain was normal (data not shown). This confirms the lack of functional redundancy for this gene in the *L. plantarum* WCFS1 genome and indicates that *melA* is essential for growth on melibiose. In analogy, quantitative determination of the bile salt hydrolase activity of the *bsh1* deletion mutant NZ5305 revealed that *bsh1* deficiency leads to a 90% reduction in bile salt hydrolase activity in *L. plantarum* WCFS1 (Fig. 5).

**Mutagenesis of genetically unlinked loci in a single genetic background.** To establish that the mutagenesis system constructed was indeed suitable for efficient construction of successive selectable-marker-free gene deletions, the *bsh1* gene was mutated in the Δ*melA* derivative (NZ5335) of *L. plantarum* WCFS1.

After transformation of the *bsh1* mutagenesis vector pNZ5325 into NZ5335 (Table 1), 38 $Cm^r$ colonies were found during primary selection of double-crossover integrants, and 4 of these colonies displayed an $Em^s$ phenotype, correlating to a *bsh1*::*lox66*-$P_{32}$-*cat-lox71* genotype (as confirmed by PCR).

Subsequently, the *lox66*-$P_{32}$-*cat*-*lox71* cassette in the Δ*melA*, *bsh1*::*lox66*-$P_{32}$-*cat*-*lox71* strain was efficiently resolved to a single in-frame *lox72* site by transient Cre expression from the erythromycin-selectable vector pNZ5348. After 72 h of incubation, all of the 15 $Em^r$ colonies that were analyzed contained cells that had undergone Cre-mediated recombination as confirmed by PCR. Following removal of the Cre expression vector by culturing without selection pressure, single-colony isolates were taken and designated NZ5337 (*L. plantarum* Δ*melA* Δ*bsh1*). Loss of the Cre expression vector and correct recombination of the target locus were confirmed by PCR and DNA sequencing of the mutated locus. Furthermore, the anticipated double-mutant (Δ*melA* Δ*bsh1*) phenotype could be confirmed, i.e., by lack of growth on melibiose as a sole carbon and energy source and strongly reduced Bsh activity (Fig. 5).

**Mutagenesis of genetically linked loci in a single genetic background.** The approaches described above showed that mutagenesis of two genetically unlinked loci (*melA* and *bsh1*) could be performed effectively. However, secondary-gene replacement at a locus that is closely linked to the initially mutated locus leads to close proximity of the *lox66*-$P_{32}$-*cat*-*lox71* cassette and the *lox72* site of the primarily targeted locus (Fig. 3), which could lead to incorrect resolution by the Cre enzyme. Provided that Cre recognizes all available *lox* sites with a certain affinity, four different modes of resolution of the locus could occur, involving the *lox66* and *lox71* sites alone or involving the *lox66* and/or *lox71* site in combination with the *lox72* site. These modes of resolution can be distinguished by PCR analysis of resulting individual clones (Fig. 3B). To evaluate the frequency of these potential artifact resolutions, a small part of the WCFS1 *lacS2* gene (lp_3486) (28), which is located directly upstream of *melA* (lp_3485) and in the same orientation as *melA*, was chosen as a secondary target for mutagenesis in the WCFS1 Δ*melA* strain NZ5335. In the presence of glucose as a carbon and energy source, mutation of *lacS2* was not expected to affect growth. The *lacS2*-specific mutagenesis vector pNZ5344 was constructed by cloning of PCR-amplified homologous DNA fragments into the mutagenesis vector pNZ5319. Following transformation of pNZ5344 into the Δ*melA* strain, double-crossover integrants were selected based on their $Cm^r$ and $Em^s$ phenotype. Out of 22 $Cm^r$ transformants, 12 clones displayed an $Em^s$ phenotype and the corresponding Δ*melA lacS2*::*lox66*-$P_{32}$-*cat*-*lox71* genotype, as could be confirmed by PCR and Southern blot analysis (Fig. 4). After Cre-mediated resolution, recombination patterns could be distinguished by PCR using primers 128, 137, and 95 in a single PCR (Table 2; Fig. 3B). Of the 192 colonies analyzed, 179 gave a PCR product, the vast majority of which (126 colonies [70.4%]) appeared to have undergone correct Cre recombination (Δ*melA* Δ*lacS2*), while almost all of the residual colonies (52 colonies [29.1%]) appeared to contain a mixed population of correctly resolved (Δ*melA* Δ*lacS2*) and unresolved (Δ*melA lacS2*::*lox66*-$P_{32}$-*cat*-*lox71*) cells. Only a single colony (0.6%) that had undergone incorrect recombination between the *lox66* site at the *lacS2* locus and the *lox72* site at the *melA* locus was detected, while no colonies that had undergone incorrect recombination between the *lox71* site at the *lacS2* locus and the *lox72* site at the *melA* locus were detected. To further establish correct Cre-mediated resolution, the genotype of a single Δ*melA* Δ*lacS2* colony (designated NZ5339) was confirmed by DNA sequencing of the mutated locus and Southern blot analysis (Fig. 4). Taken together, these experiments support the robustness and selectivity of Cre-mediated resolution of the mutant *loxP* sites introduced into the genome and exemplify the suitability of the mutagenesis system presented here for the construction of multilocus mutants in a single genetic background.

## DISCUSSION

Here we describe the construction of an effective Cre-*lox*-based toolbox for multiple gene replacements in a single genetic background. The procedure consists of three steps. In the first step, a gene-specific mutagenesis vector is constructed using standard cloning procedures. In the second step, the target gene is replaced by a *lox66*-$P_{32}$-*cat*-*lox71* cassette by double-crossover recombination. In the third step, the *lox66*-$P_{32}$-*cat*-*lox71* cassette introduced at the target locus is resolved by transient Cre expression, resulting in an in-frame *lox72* site in the genome (Fig. 1; 2C).

The mutagenesis vector presented facilitates efficient direct cloning of blunt-end PCR amplification products that represent homologous 5′- and 3′-flanking regions of any desired target locus in rare-cutting blunt-end restriction sites in the medium-copy-number *E. coli* cloning vector. The presence of two selectable-marker gene cassettes on the mutagenesis vector enables direct selection of double-crossover integrants. Direct selection of mutants provides a major advantage in procedures aiming to generate gene mutations that might result in growth retardation, where mutants may not be obtained when a method that implements a single selectable marker on the mutagenesis vector (i.e., pUC18 or $pG^+$host) is used (12, 64). However, the presence of a selectable marker in the chromosome hampers multiple gene replacements and may influence the expression of surrounding genes. As opposed to most mutagenesis systems that enable direct selection of double-crossover mutants by implementation of two selectable markers on the mutagenesis vector (such as pUC18ery) (60), the method described here adds the option of selectable-marker removal from the genome by transient Cre expression in the gene replacement background using an unstable and easily curable Cre-expressing vector that ensures the removal of Cre activity before the introduction of additional mutations. Thus, our system provides an important advantage over mutagenesis systems for gram-positive bacteria that have been described to date.

Targeting of the *melA* and *bsh1* locus of the gram-positive model organism *L. plantarum* WCFS1 (28) showed the effectiveness of our system for construction of single-locus double-crossover mutants and subsequent marker removal. The *melA* gene was shown to be essential for the growth of *L. plantarum* WCFS1 on melibiose as a sole carbon and energy source. Although predicted to be fourfold redundant, the *bsh1* gene appeared to be the major bile salt hydrolase-encoding gene in *L. plantarum* WCFS1. This is in good agreement with the finding that *L. plantarum* WCFS1 *bsh1* is almost identical to the *bsh* gene of *L. plantarum* LP80, for which a previously constructed mutant was shown to be bile salt hydrolase deficient (35). However, to determine whether *bsh2*, *bsh3*, and/or

*bsh4* plays a role in the remaining Bsh activity in the Δ*bsh1* derivative of strain WCFS1, further investigation is required.

Furthermore, successful generation of multiple gene deletions by Cre-*lox* recombination depends on correct resolution events even in the presence of previously integrated *lox72* sites. In the work presented here, the efficiency of Cre-*lox*-based removal of the $P_{32}$-*cat* selectable-marker cassette in multiple gene replacements was determined by successive mutagenesis of genetically unlinked and closely linked chromosomal loci. Thus, *bsh1*was effectively deleted in an *L. plantarum* WCFS1 Δ*melA* strain, resulting in a Δ*melA* Δ*bsh1* double-mutant strain, which displayed the anticipated combination of the single-locus mutant phenotypes. During successive mutagenesis of the genetically closely linked loci *melA* and *lacS2* in *L. plantarum* WCFS1 (Fig. 3), Cre-mediated resolution of *lox66* and *lox71* in close proximity to a *lox72* site was shown to occur correctly in 99.4% of the colonies that were successfully analyzed. Moreover, no colonies were found to have undergone incorrect resolution of *lox71* and *lox72*. However, the latter type of recombination (*lox71* and *lox72*) results in a *lox71* site, which in turn can recombine with the still remaining *lox66* site, thereby generating a single *lox72* site (Fig. 3B). This situation is indistinguishable from direct recombination of *lox66* and *lox72*, which was found to occur in only a single colony tested (0.6%). The fact that correct Cre resolution of *lox66* and *lox71* occurs almost exclusively even in close proximity to a *lox72* site emphasizes the selectivity of the Cre enzyme and underlines the advantage of this system relative to mutagenesis systems that employ native *loxP* sites, which have been used for some gram-negative bacteria (41, 46, 49). Especially for prokaryotes, where only one chromosome is present, the use of *loxP* sites to resolve the selectable-marker cassette in multiple gene deletions is highly undesirable, because it can lead to (large) genomic inversions and/or rearrangements (14, 15, 57).

Although the Cre-*lox* system as described here was used for *L. plantarum* WCFS1, it can easily be adapted to other gram-positive bacteria, including other lactic acid bacteria such as *Lactococcus lactis* and *Streptococcus thermophilus*. The only prerequisite for the use of this system is the availability of a method to establish transient Cre expression. For example, a temperature-sensitive vector such as $pG^+$host (40) can be used for this purpose by subcloning of the $P_{1144}$-Cre expression cassette present in pNZ5348. The applicability of this approach for *L. lactis* has already been established in our laboratory (R. Brooijmans, personal communication). More-advanced alternative possibilities could also be employed, including, for example, strictly controlled Cre expression from a permanently present plasmid or from a chromosomal locus, or expression of Cre from a plasmid in which Cre or the origin of replication is flanked by *lox* sites to ensure cessation of Cre activity before construction of additional mutations in the same genetic background. Analogously, the pNZ5319 mutagenesis vector could be modified by replacement of the pACYC184 replicon with a temperature-sensitive replicon ($pG^+$host derived) in order to facilitate mutagenesis in bacterial species with low transformation frequencies that preclude the suicide mutagenesis strategy employed here. These relatively simple modifications allow functional implementation of the Cre-*lox*-based mutagenesis system with a range of other gram-positive or gram-negative bacteria and exemplify the broad applicability of the system presented here.

In conclusion, the multiple gene deletion system for gram-positive bacteria presented here allows for effective, standardized construction of double-crossover mutants that can be introduced in a single genetic background by a simple repetitive procedure using mutant *lox* sites for Cre-mediated selectable-marker removal.

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